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Phylogenetic relationships and reclassification of *Spirosphaera lignicola*, an enigmatic aeroaquatic fungus

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Abstract — The enigmatic *Spirosphaera lignicola* is revised based on recent collections from Austria. The taxon does not fit the generic circumscription of the genus *Spirosphaera* in its morphological features but clearly belongs to *Dendroclathra*. Nuclear ITS rDNA sequence data from recent collections and the type specimens indicate conspecificity of *S. lignicola* with *Dendroclathra caeruleofusca*. *Spirosphaera lignicola* is transferred to the genus *Dendroclathra*, and a recent collection with an ITS sequence identical to the type specimen is designated as epitype. Morphology is illustrated with SEM and LM pictures. Phylogenetic analyses of LSU, ITS and *tef1* sequence data reveal a phylogenetic affinity of *Dendroclathra* to the *Microascales* (*Hypocreomycetidae*), being therefore phylogenetically distant from *Spirosphaera floriformis*, the generic type, which belongs to the *Leotiomycetes*. The recent collections are the first records of this species for Austria. The distribution and aeroaquatic ecology of *D. lignicola* are briefly discussed.

Key words — anamorph, ascomycetes, freshwater fungi, molecular phylogeny, taxonomy

Introduction

Spirosphaera lignicola was described from deciduous wood submerged in a stream in Italy (Abdullah et al. 1998). Although the description and illustrations indicated that *S. lignicola* does not fit the morphological generic circumscription of *Spirosphaera* Beverw. due to the lack of distinct coiling of the conidial filament and of the unilateral branching pattern typical of *Spirosphaera* (Hennebert 1998), no suitable alternative classification could be proposed with the data at hand (Voglmayr 2004). No type culture was deposited in a culture collection, and the sporulation on the holotype specimen (dried malt extract agar culture) did not reveal features enabling an appropriate generic classification. It was therefore considered an enigmatic fungus requiring additional studies.

Dendroclathra Voglmayr & G. Delgado is a monotypic anamorph genus, with *D. caeruleofusca* as the type species (Voglmayr & Delgado-Rodríguez

2001). The diagnostic features of the genus were compact multicellular globose conidia, formed by a conidial filament first branching extensively tree-like and subsequently many times irregularly dichotomously in a tangential manner, the tips frequently fusing with neighboring cells upon mutual contact. Its multicellular conidia are therefore morphologically quite complex. *Dendroclathra caeruleofusca* was collected and isolated from a dead bamboo stem submerged in a river in tropical Cuba. This habitat, together with its conidial morphology, indicated an aeroaquatic ecology (Voglmayr & Delgado-Rodríguez 2001). Although it could be easily isolated in pure culture and grew vigorously on PDA and MEA, no sporulation was observed on agar cultures but only on natural substrate. No connection with *Spirosphaera lignicola* was made at that time despite some superficial similarity, and according to the description and the SEM pictures, the latter apparently lacked the very prominent fusion of the conidial cells. The conidia present on the holotype of *S. lignicola*, which consists of a dried culture, showed a different, more irregular branching pattern not in line with the more complex conidia of *D. caeruleofusca*, which were only known from natural substrate.

During recent research on biodiversity and phylogeny of aeroaquatic fungi, a fungus was repeatedly collected and isolated in the surroundings of Vienna from wood submerged in various small streams, ditches, and oxbow lakes, which closely matched *Dendroclathra caeruleofusca* in conidial ontogeny and morphology. However, unlike the type culture of the latter, in some isolates atypical but abundant conidia were formed on agar cultures, which closely resembled those present on the dried holotype culture of *Spirosphaera lignicola*. To elucidate whether *S. lignicola* and *D. caeruleofusca* are congeneric or even conspecific and to which species the Austrian collections belong, detailed DNA and morphological studies were performed.

Materials & methods

Sampling and documentation

Submerged, rotting twigs were taken from various small rivulets, ditches and oxbow lakes, packed into plastic bags and returned to the laboratory. The twigs were cut into pieces, rinsed in tap water and spread on Petri dishes lined with moist filter paper, kept damp and exposed to natural light at room temperature. The Petri dishes were regularly examined under a dissecting microscope for the presence of propagules of aeroaquatic fungi. After spores had developed, they were transferred aseptically to 2% malt extract agar (MEA) plates, where they readily germinated. Spores from both natural substrate and agar cultures were examined under the light microscope. Twig fragments containing spores and sporulating agar cultures were air-dried and deposited in the herbarium of the Faculty Center of Biodiversity of the University of Vienna (WU). Living cultures were deposited in CBS (Utrecht, The Netherlands). The collection data of the specimens investigated are listed in the Taxonomy section.

Light microscopy

Conidia from fresh and dried specimens were mounted in tap water or L4 (general mounting fluid after Clémençon 1972) for light microscopy. Slides were examined and photographed using a Zeiss Axio Imager.A1 (Zeiss, Jena, Germany) microscope equipped with a Zeiss AxioCam ICc3 digital camera.

Scanning electron microscopy

For scanning electron microscopy (SEM), spores grown on natural substrate were prepared according to the method described in Halbritter (1998). After preparation, the specimens were mounted on Cambridge stubs, sputter coated with gold, and examined in a Jeol T 300 scanning electron microscope at 10 kV.

DNA extraction, PCR and sequencing

For sample preparation from living cultures, small pieces were cut out at the margin of actively growing agar cultures, put into 2 ml reaction-tubes, immediately frozen, and freeze-dried. Alternatively, liquid cultures were grown in 100 ml 1% aqueous malt extract solution in 250 ml flasks, immediately frozen after harvesting, and freeze-dried. After addition of several glass beads (ca. 2 mm diameter), the samples were ground in a Retsch 200 mixer mill for 10 minutes. Subsequently, DNA was extracted using the modified CTAB-protocol described in Riethmüller et al. (2002).

For DNA extraction from the type specimen of *Spirosphaera lignicola* (IMI 375019), a small piece (ca. 2×2 mm) containing only mycelium immersed in agar was cut from the margin of the dried culture using a sterile scalpel and forceps and placed in a 1.5 ml reaction-tube. Then, 300 µl CTAB buffer and quartz sand were added, the samples incubated in a thermo block for about 10 minutes at 65 °C and subsequently thoroughly ground using a conical micro pestle. Afterwards, the sample was again incubated for additional 50 minutes at 65 °C and further processed like the samples from living cultures.

The complete ITS rDNA region was amplified with primers ITS4 and ITS5 (White et al. 1990); the D1, D2 region of the nuLSU rDNA region with primers LR0R (Rehner & Samuels 1994) and TW14 (White et al. 1990). Alternatively, a ca. 1550 bp fragment containing partial SSU rDNA, ITS1, 5-8S rDNA, ITS2 and partial nuLSU was amplified with primers V9G (de Hoog & Gerrits van den Ende 1998) and LR5 (Vilgalys & Hester 1990). A ca. 1200 bp fragment of the *tef1* (translation elongation factor 1 alpha) gene was amplified with primers EF1728F (Chaverri & Samuels 2003) and TEF1LLErev (Jaklitsch et al. 2006). The latter fragment includes the fourth and the fifth intron and a significant portion of the last large exon. PCR products were purified either by gel electrophoresis in combination with the QIAquick Kit (Qiagen) according to the manufacturer's instructions or an enzymatic PCR cleanup as described in Voglmayr & Jaklitsch (2008). DNA was cycle-sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems, Warrington) with primers ITS4, ITS5, LR0R, TW14, V9G, LR3 (Vilgalys & Hester 1990), or LR5, depending on the PCR fragment, and an automated DNA sequencer (ABI 377 or 3130xl Genetic Analyzer, Applied Biosystems). The GenBank accession numbers of the sequences obtained in the present study are listed in the Taxonomy section.

Data analysis

For the nuLSU rDNA analysis, first a blast search in the GenBank sequence database was performed. As the sequences most similar to *Dendroclathra* all were from *Hypocreomycetidae*, representative sequences were selected according to Zhang et al. (2006) and downloaded from GenBank. The GenBank accession numbers of the selected sequences are given in the tree, following the taxon names (Fig. 1). The nuLSU sequence alignment was produced with Muscle version 3.7 (Edgar 2004) and visually checked with BioEdit (Hall 1999), version 7.0.9.0.

Nucleotide blast searches were done with the complete ITS rDNA region as well as the exon of the *tef1* gene the of *Dendroclathra lignicola* to reveal the closest sequences. A representative taxonomic selection of sequences (Table 1) were aligned with Muscle to obtain a representative matrix for phylogenetic analysis.

Table 1. Taxa and GenBank accession numbers of ITS and *tef1* sequences selected for phylogenetic analyses.

Species	Sequences	
	ITS	*tef1*
Ambrosiella xylebori Arx & Hennebert		DQ471102
Aniptodera chesapeakensis Shearer & M.A. Mill.		DQ471064
Balansia henningsiana (Möller) Diehl		AY489610
Ceratocystis polonica (Siemaszko) C. Moreau	AY233899	
Chaetosphaeria tulasneorum Réblová & W. Gams	AF178547	
Claviceps purpurea (Fr.) Tul.		AF543778
Coniochaeta sp.	AM262406	
Cordyceps bassiana Z.Z. Li et al.	AB237657	
Doratomyces stemonitis (Pers.) F.J. Morton & G. Sm.		DQ836916
Elaphocordyceps capitata (Holmsk.) G.H. Sung et al.		AY489615
E. inegoensis (Kobayasi) G.H. Sung et al.	AB027368	
E. ophioglossoides (Ehrh.) G.H. Sung et al.		AY489618
Fimetariella rabenhorstii (Niessl) N. Lundq.	EU781677	
Glomerella cingulata (Stoneman) Spauld. & H. Schrenk		AF543773
G. lindemuthiana Shear	EU400135	
G. tucumanensis (Speg.) Arx & E. Müll.	AY944753	
Graphium basitruncatum (Matsush.) Seifert & G. Okada	EF165016	
G. penicillioides Corda		DQ471110
Hypocrea americana (Canham) Overton		DQ471043
Lasiosphaeria sorbina (Nyl.) P. Karst.	AY587934	
Microascus longirostris Zukal		DQ836913
M. trigonosporus C.W. Emmons & B.O. Dodge		DQ471077
Myrothecium cinctum (Corda) Sacc.	AJ302004	
M. roridum Tode		AY489603
Nectria cinnabarina (Tode) Fr.		AF543785
N. mauritiicola (Henn.) Seifert & Samuels	AY138847	
Niesslia exilis (Alb. & Schwein.) G. Winter		AY489614
Peethambara spirostriata (Rossman) Rossman		AY489619
Petriella setifera (Alf. Schmidt) Curzi		DQ836911
Porosphaerella cordanophora E. Müll. & Samuels	AF178563	
Pseudallescheria boydii (Shear) McGinnis et al.	AY228112	
Scedosporium prolificans (Hennebert & B.G. Desai) E. Guého & de Hoog	AY228120	
Verticillium dahliae Kleb.		AY489632
Xylaria acuta Peck (outgroup)		DQ471048
X. hypoxylon (L.) Grev. (outgroup)		DQ471042
X. longipes Nitschke (outgroup)	AY909016	
X. mali Fromme (outgroup)	AF163040	

Maximum parsimony (MP) analyses were performed with PAUP*4.0b10 (Swofford 2002), using 1000 replicates of heuristic search with random addition of sequences and subsequent TBR branch swapping (MULTREES option in effect, steepest descent option not in effect, COLLAPSE=maxbrlen). Maximum parsimony bootstrap analyses were done with the same settings, with ten rounds of random sequence addition and subsequent TBR branch swapping during each bootstrap replicate. Gaps were treated as missing data.

Results

Sequence similarity of *Dendroclathra caeruleofusca* and *Spirosphaera lignicola*

The complete ITS rDNA sequences of the types of *Dendroclathra caeruleofusca* and *Spirosphaera lignicola* as well as of all Austrian accessions isolated during the present study were highly similar (sequence similarities of 98.8–100%; sequence differences of 0–6 nucleotides including gaps). The ITS sequence of the type of *S. lignicola* was identical to one Austrian collection (A270), whereas the type of *D. caeruleofusca* from Cuba was most similar to another Austrian collection (A266), from which it differed by two nucleotide substitutions. The *tef1* sequences were also highly similar (sequence similarities of 98.8–100%); however, the type of *D. caeruleofusca* differed from the Austrian collections by 22–23 nucleotides (almost all in introns 4 and 5), whereas the Austrian collections showed only 0–6 nucleotide differences from each other. For the type of *S. lignicola*, no *tef1* sequence was available. All nuLSU sequences of *Dendroclathra* revealed were identical.

Phylogenetic relationships

After exclusion of ambiguously aligned regions, 1573 characters of the resulting partial nuLSU alignment were included in the subsequent phylogenetic analyses. Of these, 1030 characters were constant; of the variable characters 144 were parsimony-uninformative and 399 parsimony-informative. The MP analyses of the nuLSU rDNA alignment revealed one most parsimonious tree of score 1743 (Fig. 1). The topology of the tree is similar to that of Zhang et al. (2006); however, most of the tree backbone lacked significant bootstrap support. *Dendroclathra* was embedded within a paraphyletic *Microascales*, also without support. *Spirosphaera floriformis*, the generic type, was placed within *Leotiomycetes* with maximum support (Fig. 1).

In the nucleotide blast search, no ITS sequences highly similar to *Dendroclathra* could be retrieved; the most similar taxonomically identified sequences belonged to members of *Hypocreales*, *Microascales* and *Glomerellaceae*. The sequence alignment of these most similar ITS sequences contained numerous indels and ambiguously aligned positions within both the ITS1 and ITS2 regions. After exclusion of these regions, 650 characters remained for a phylogenetic analysis, of which 300 characters were constant, 117 variable characters parsimony-

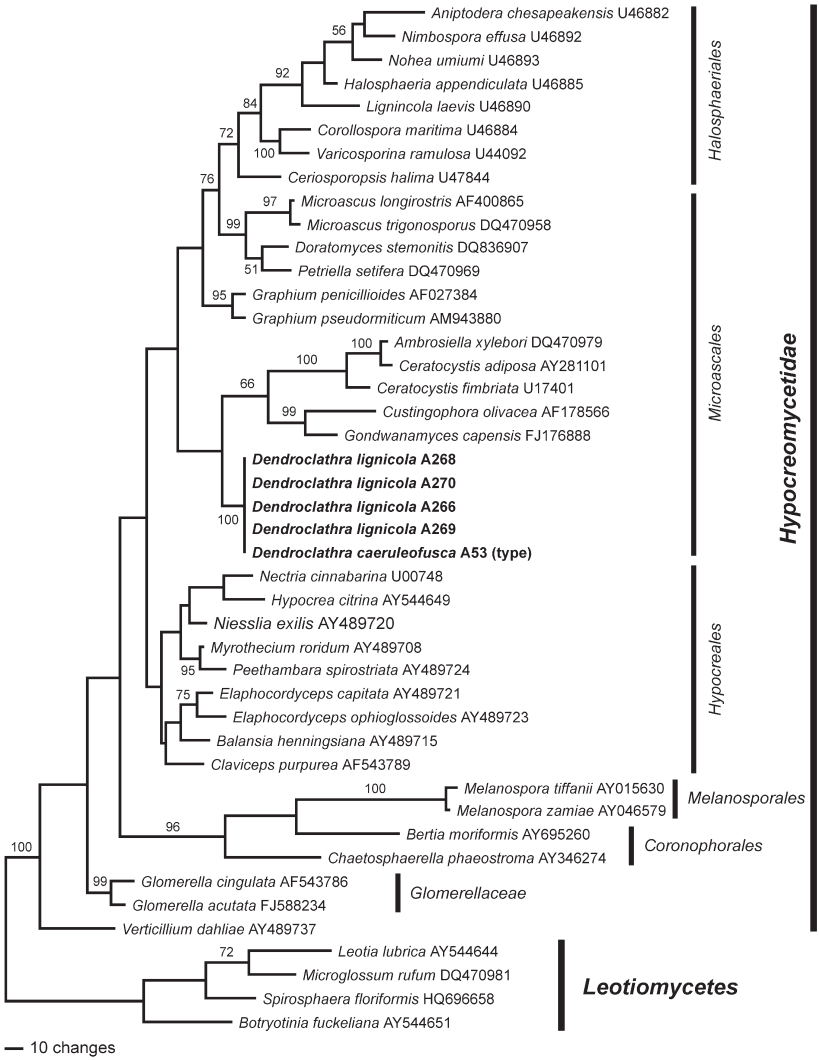


Fig. 1. Phylogram of the MP tree revealed by PAUP from an analysis of the nuLSU rDNA matrix of selected *Hypocreomycetidae* and *Leotiomycetes*, showing the phylogenetic affinities of *Dendroclathra* to the *Microascales*. GenBank sequence accession numbers follow the taxon names. MP bootstrap support above 50% is given above or below the branches. Labels in bold denote sequences obtained during the present study.

uninformative and 233 parsimony-informative. The MP analysis revealed nine most parsimonious trees; in all of these trees, *Dendroclathra* was sister group of the *Microascales* clade (*Scedosporium prolificans*, *Pseudallescheria boydii*,

Ceratocystis polonica, Graphium basitruncatum), however, without bootstrap support (data not shown).

For the *tef1* gene, a nucleotide blast search revealed members of *Hypocreales*, *Microascales* and *Glomerellaceae* as closest relatives as well. Of the altogether 1018 nucleotide characters included in the MP analysis, 654 characters were constant, 86 variable characters parsimony-uninformative and 278 parsimony-informative. MP analysis revealed a single most parsimonious tree, which revealed *Dendroclathra* as sister group to *Graphium penicillioides* (*Microascales*) with moderate bootstrap support (78%; data not shown).

Morphology

The cultures of the Austrian isolates produced conidia very similar to those of the dried type culture of *Spirosphaera lignicola*. Conidia in culture differed from those on natural substrate in being simpler, i.e. the conidial filaments are rather loosely branched tree-like and less tightly interwoven (Fig. 2a–d), lacking the tangential branching of tightly packed conidial filaments typical for older conidia on natural substrate. Therefore, also the typical fusion of conidial cells upon mutual contact (Figs. 2g, 3j) is not observed in conidia produced in pure culture. Anisotomous-dichotomous branching of the conidial filament is present in conidia from pure culture (Fig. 2a) as well as from natural substrate (Fig. 2e), but mostly less apparent and regular in conidia from pure culture (Fig. 2b–d). The conidia of the Austrian collections from natural substrate matched those of the type collection of *Dendroclathra caeruleofusca* (compare Figs. 2e–g, 3 with Voglmayr & Delgado-Rodríguez 2001: figs. 1–14). Upon higher magnification, in SEM the conidial cells are verruculose (Fig. 3k).

Taxonomy

Dendroclathra lignicola (Abdullah, Gené & Guarro) Voglmayr, **comb. nov.**

MycoBank MB 519527 Figs. 2, 3

≡ *Spirosphaera lignicola* Abdullah, Gené & Guarro, Mycotaxon 66: 268 (1998).

= *Dendroclathra caeruleofusca* Voglmayr & G. Delgado, Can. J. Bot. 79: 997 (2001).

Type — **ITALY**. **Siena**: Monticiano, on submerged twig in a stream, 5 Nov. 1996 S.K. Abdullah (IMI 375019, **holotype** of *Spirosphaera lignicola*; ex-type ITS sequence: EU873531). **AUSTRIA**. **Wien**: Ottakring, Moosgraben, on wood of deciduous tree submerged in small rivulet, 10 Apr. 2006 A270 (WU 31301 **epitype here designated**, ex-epitype culture CBS 122534; ex-epitype sequences: ITS-LSU: EU873530, *tef1*: EU873534)

Discussion

This is the first time that sequence data are available for *Spirosphaera lignicola* and *Dendroclathra caeruleofusca*. The molecular data indicate that both species are conspecific, as their LSU sequences are identical, and there are only few nucleotide substitutions in the ITS. However, in the *tef1* sequences,

the Cuban isolate of *D. caeruleofusca* deviates significantly from the European isolates, which corroborates some genetic differentiation between European and American isolates indicative of geographic isolation. Conspecificity of the Austrian isolates with the type of *S. lignicola* from Italy is also corroborated by the similarity of conidia produced in pure culture. However, the conidia produced in pure culture deviate from those produced on natural substrate in being less complex and atypical, which demonstrates that morphological species identification based on conidia can be difficult in case of aberrant conidiation in pure culture. *Spirosphaera lignicola* has been described solely from conidia produced in pure culture. Conspecificity with *D. caeruleofusca* was therefore not apparent, as the species description and illustrations of *S. lignicola* were incomplete, and conidiation was absent in the type culture of *D. caeruleofusca*.

Conidial morphology of *S. lignicola* does not fit the genus *Spirosphaera*. In *S. lignicola*, the conidial filaments are never coiled which in combination with unilateral branching, is the main diagnostic feature of *Spirosphaera* (Hennebert 1998). In addition, the cells of the conidial filament of *S. lignicola* are different in being mostly isodiametric and commonly fusing upon mutual contact, whereas they are elongated and never fusing in *Spirosphaera* (Voglmayr & Delgado-Rodríguez 2001). In addition, *S. lignicola* is placed within *Hypocreomycetidae* (Fig. 1) and therefore phylogenetically distinct from the generic type of *Spirosphaera*, which belongs to the *Leotiomycetes* (Fig. 1; Voglmayr 2004). As *S. lignicola* is apparently conspecific with *D. caeruleofusca* and has priority, it has to be transferred to *Dendroclathra*, *D. caeruleofusca* becoming a synonym of *D. lignicola*.

Concerning conidial morphology, *Dendroclathra* is a typical member of the aeroaquatic fungi (Voglmayr & Delgado-Rodríguez 2001) in having buoyant multicellular conidia enclosing air between their cells. The ecology of the Austrian collections is similar to the Italian and Cuban collections, which also grew on wood submerged in small streams. This habitat is rather atypical for aeroaquatic fungi, which are mostly found on litter submerged in stagnant water bodies characterized by oxygen depletion (Michaelides & Kendrick 1981, Webster & Descals 1982). Conversely, the habitats of the Austrian collections appeared to be well aerated, lacking the sulfide smell typical of most aeroaquatic habitats. This may be the reason why it has only rarely been collected and not previously recorded for Austria despite the extensive investigations on aeroaquatic fungi by the author. It may be a rather common species in some areas, as it has been found at several localities in the vicinity of Vienna and may have a wide distribution in suitable habitats, at least in the northern hemisphere.

Additional specimens examined — **AUSTRIA**. **Niederösterreich**: Mödling, Gießhübl, on wood of deciduous tree submerged in small rivulet, 2 Apr. 2006 H. Voglmayr A268 (WU 31302; culture deposited as CBS 122533; sequences: ITS-LSU:

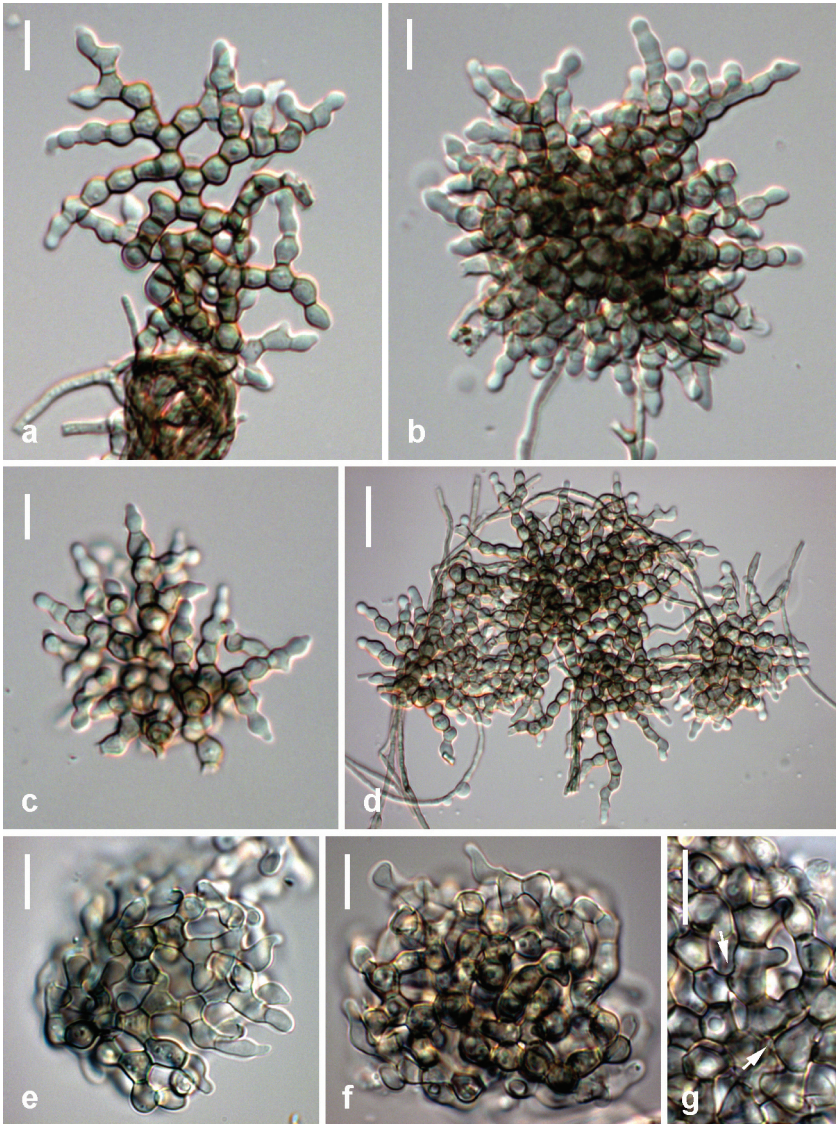
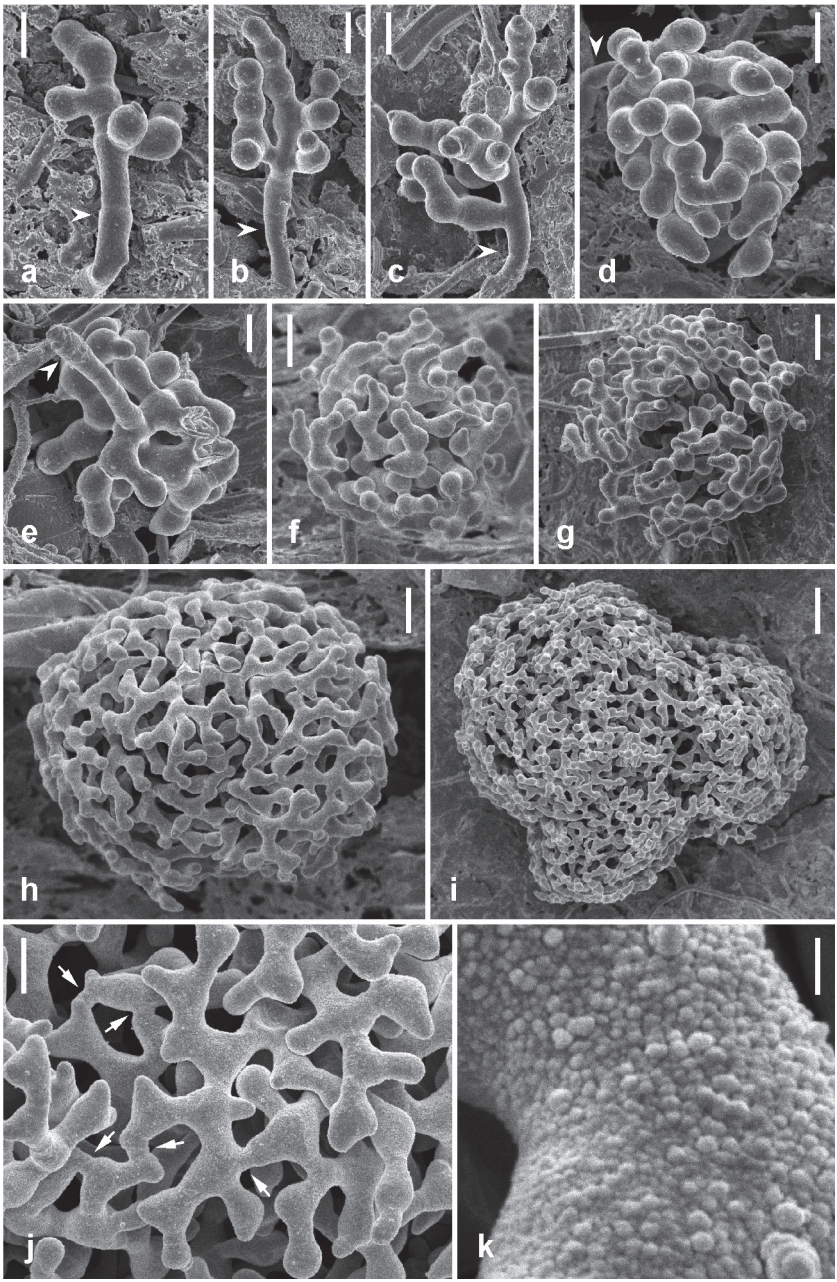


Fig. 2. LM of *Dendroclathra lignicola*. a–d. Conidia produced on 2% malt extract agar, showing the anisotomously dichotomous branching of the conidial filament, but no tangential branching and without fusion of filament cells upon mutual contact. e–f. Immature conidia from natural substrate in side view (e) and from above (f); in (e) showing the anisotomously dichotomous branching of the conidial filament. g. Detail from squash mount of mature conidium from natural substrate, showing densely packed conidial filament fusing upon mutual contact (white arrows). Sources: a–d. IMI 375019 (type), e–g. WU 31304. Scale bars: a–c, e–g = 10 µm, d = 20 µm.


a
b
c
d
e
f
g
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k

EU873529, *tef1*: EU873536); Baden, Helenental, Cholerakapelle, on wood of deciduous tree submerged in small rivulet, 23 Apr. 2006 H. Voglmayr A269 (WU 31303, culture deposited as CBS 122531; sequences: ITS-LSU: EU873528, *tef1*: EU873535). **Wien**: Donaustadt, Lobau, Panozzalacke, on wood of deciduous tree submerged in an oxbow lake, 8 Apr. 2006 H. Voglmayr A266 (WU 31304, culture deposited as CBS 122532; sequences: ITS-LSU: EU873527, *tef1*: EU873533); Donaustadt, Lobau, Mühlleitener Furt, on wood of deciduous tree submerged in an oxbow lake, 21 Apr. 2006 H. Voglmayr A267 (WU 31305, culture deposited as CBS 122530; sequence: *tef1*: EU873537). **CUBA**. **Pinar del Río**: Sierra del Rosario Biosphere Reserve, San Juan River, on submerged bamboo twig, 20 Sep. 2000 G. Delgado-Rodríguez (WU 21361, **isotype** of *Dendroclathra caeruleofusca*; ex-holotype sequences: ITS-LSU: EU873526, *tef1*: EU873532).

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Fig. 3. SEM of conidia of *Dendroclathra lignicola* (WU 31305). Arrows denote conidiophores. a–c. Young conidia on conidiophores, showing initial tree-like branching. d, e. Young conidia from above (d) and below (e). f–g. Immature conidia from above. h–i. Mature conidia from above showing dense tangential branching. j. Detail of mature conidium, showing cell fusion upon mutual contact (arrows). k. Conidial cells with verruculose surface ornamentation. Scale bars: a–e, j = 5 µm f–h = 10 µm, i = 20 µm, k = 0.5 µm.

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